

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number  
WO 02/081721 A2(51) International Patent Classification<sup>7</sup>: C12P 13/00

(21) International Application Number: PCT/EP02/02419

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
101 16 518.8 3 April 2001 (03.04.2001) DE

GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

## Published:

— without international search report and to be republished upon receipt of that report

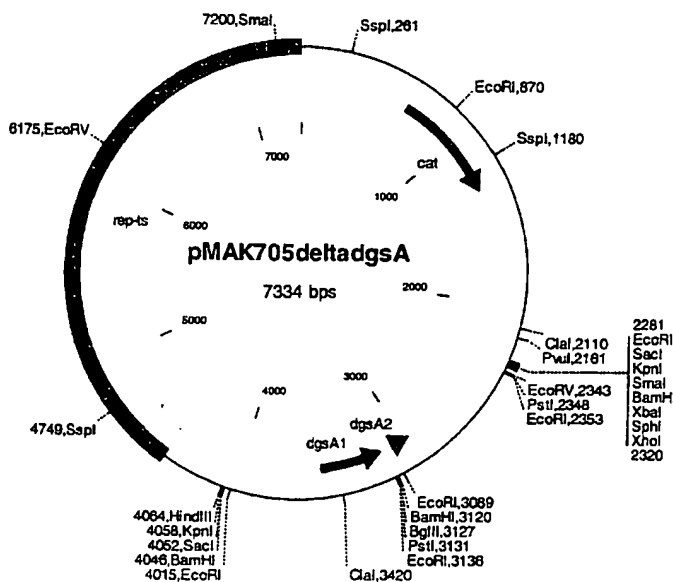
(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1, 33619 Bielefeld (DE). HERMANN, Thomas; Zirkonstrasse 8, 33739 Bielefeld (DE).

— with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE THAT CONTAIN AN ATTENUATED DGS A GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: (a) cultivation of a bacterium of the family Enterobacteriaceae, which contains an attenuated dgsA gene, in a medium in which the L-amino acid is present, in which the dgsA gene is expressed, (b) isolation of the L-amino acid from the medium or in the cells of the bacteria, and (c) isolation of the L-amino acid.

**Process for the Production of L-Amino Acids Using  
Strains of the Family Enterobacteriaceae  
that Contain an Attenuated dgsA Gene**

Field of the Invention

- 5 The present invention relates to a process for the enzymatic production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae in which the dgsA gene is attenuated.

Prior Art

- 10 L-amino acids, in particular L-threonine, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry, and most especially in animal nutrition.

- It is known to produce L-amino acids by fermentation of  
15 strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. On account of their great importance efforts are constantly being made to improve processes for producing the latter. Process improvements may relate to fermentation technology  
20 measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form, for example by ion exchange chromatography, or the intrinsic  
25 performance properties of the microorganism itself.

- Methods comprising mutagenesis, selection and mutant choice are employed in order to improve the performance properties of these microorganisms. In this way strains are obtained that are resistant to antimetabolites, such as for example  
30 the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV) or are auxotrophic for regulatorily important metabolites,

and that produce L-amino acids such as for example L-threonine.

Methods of recombinant DNA technology have also been used for some years in order to improve strains of the family  
5 Enterobacteriaceae producing L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating their effect on production.

#### Object of the Invention

The object of the invention is to provide new measures for  
10 the improved enzymatic production of L-amino acids, in particular L-threonine.

#### Summary of the Invention

The present invention provides a process for the enzymatic production of L-amino acids, in particular L-threonine,  
15 using microorganisms of the family Enterobacteriaceae that in particular already produce L-amino acids and in which the nucleotide sequence coding for the dgsA gene is attenuated.

#### Detailed Description of the Invention

20 Where L-amino acids or amino acids are mentioned hereinafter, this is understood to mean one or more amino acids including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-  
25 methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-threonine is particularly preferred.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of  
30 one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by using for example a weak

promoter or a gene or allele that codes for a corresponding enzyme with a low activity and/or that inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

- 5 By means of these attenuation measures the activity or concentration of the corresponding protein is generally reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, or the activity or concentration of the protein in  
10 the initial microorganism.

The process is characterized in that the following steps are carried out:

- 15 a) fermentation of microorganism of the family Enterobacteriaceae in which the *dgsA* gene is attenuated,
- b) enrichment of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and
- 20 c) isolation of the desired L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or parts thereof remain in the product.

The microorganisms that are the subject of the present invention can produce L-amino acids from glucose, sucrose,  
25 lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. The microorganisms are members of the family Enterobacteriaceae selected from the genera *Escherichia*, *Erwinia*, *Providencia* and *Serratia*. The genera *Escherichia* and *Serratia* are  
30 preferred. In the case of the genus *Escherichia* the species *Escherichia coli* may in particular be mentioned, and in the case of the genus *Serratia* the species *Serratia marcescens* may in particular be mentioned.

Suitable strains of the genus *Escherichia*, in particular those of the species *Escherichia coli*, that produce in particular L-threonine include for example:

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132

Suitable strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, that produce L-threonine  
15 include for example:

- Serratia marcescens HNr21
- Serratia marcescens TLR156
- Serratia marcescens T2000

Strains of the family of Enterobacteriaceae producing L-  
20 threonine preferably have, *inter alia*, one or more of the genetic or phenotype features selected from the following group: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic  
25 acid, resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues such as for example valine hydroxamate, resistance to purine analogues such as for example 6-dimethylaminopurine, need for L-methionine, optionally  
30 partial and compensatable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

- resistance to L-aspartate, resistance to L-leucine,  
resistance to L-phenylalanine, resistance to L-serine,  
resistance to L-cysteine, resistance to L-valine,  
sensitivity to fluoropyruvate, defective threonine  
5 dehydrogenase, optionally ability to utilise sucrose,  
enhancement of the threonine operon, enhancement of  
homoserine dehydrogenase, L-aspartate kinase I, preferably  
of the feedback-resistant form, enhancement of homoserine  
kinase, enhancement of threonine synthase, enhancement of  
10 aspartate kinase, optionally of the feedback-resistant  
form, enhancement of aspartate semialdehyde dehydrogenase,  
enhancement of phosphoenol pyruvate carboxylase, optionally  
of the feedback-resistant form, enhancement of phosphoenol  
pyruvate synthase, enhancement of transhydrogenase,  
15 enhancement of the RhtB gene product, enhancement of the  
RhtC gene product, enhancement of the YfiK gene product,  
enhancement of a pyruvate carboxylase, and attenuation of  
acetic acid formation.

- It has now been found that microorganisms of the family  
20 Enterobacteriaceae after attenuation, in particular after  
switching off the *dgsA* gene, produce L-amino acids, in  
particular L-threonine, in an improved way.

- The nucleotide sequences of the *Escherichia coli* genes  
belong to the prior art and may also be obtained from the  
25 genome sequence of *Escherichia coli* published by Blattner  
et al. (Science 277, 1453 - 1462 (1997)).

The *dgsA* gene is described *inter alia* by the following  
data:

- Designation: Regulator of the phosphotransferase system  
30 EC-No.: -  
Reference: Hosono et al.; Bioscience, Biotechnology  
and Biochemistry 59, 256-261 (1995) Morris  
et al.; Journal of Bacteriology 163, 785-786  
(1985)

Accession No.: AE000255

Note: The *dgsA* gene is also designated as *mlc* gene in the prior art.

Apart from the described *dgsA* gene, alleles of the gene may  
5 be used that result from the degeneracy of the genetic code or from functionally neutral sense mutations, the activity of the protein not being substantially altered.

In order to achieve an attenuation the expression of the gene or the catalytic properties of the enzyme proteins may  
10 for example be reduced or switched off. Optionally both measures may be combined.

The gene expression may be reduced by suitable culture conditions, by genetic alteration (mutation) of the signal structures of the gene expression, or also by antisense-RNA  
15 techniques. Signal structures of the gene expression are for example repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start codon and terminators. The person skilled in the art may find relevant information in, *inter alia*, articles by  
20 Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), by Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999), Franch and Gerdes (Current Opinion in Microbiology 3, 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as for  
25 example the textbook by Knippers ("Molekulare Genetik", 6<sup>th</sup> Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene and Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change or reduction of the  
30 catalytic properties of enzyme proteins are known from the prior art. As examples there may be mentioned the work by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences USA 95, 5511-5515 (1998), Wente and

Schachmann (Journal of Biological Chemistry 266, 20833-20839 (1991)). Detailed information may be obtained from known textbooks on genetics and molecular biology, such as for example that by Hagemann ("Allgemeine Genetik", Gustav  
5 Fischer Verlag, Stuttgart, 1986).

Suitable mutations include transitions, transversions, insertions and deletions. Depending on the action of the amino acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or  
10 deletions of at least one base pair in a gene lead to frame shift mutations, which in turn lead to the incorporation of false amino acids or the premature termination of a translation. If as a result of the mutation a stop codon is formed in the coding region, this also leads to a  
15 premature termination of the translation. Deletions of several codons typically lead to a complete disruption of the enzyme activity. Details regarding the production of such mutations belong to the prior art and may be obtained from known textbooks on genetics and molecular biology,  
20 such as for example the textbook by Knippers ("Molekulare Genetik", 6<sup>th</sup> Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag,  
25 Stuttgart, 1986).

Suitable mutations in the genes such as for example deletion mutations may be incorporated by gene and/or allele exchange in suitable strains.

A conventional method is the method of gene exchange by  
30 means of a conditionally replicating pSC101 derivate pMAK705 described by Hamilton et al. (Journal of Bacteriology 171, 4617 - 4622 (1989)). Other methods described in the prior art, such as for example that of Martinez-Morales et al. (Journal of Bacteriology 181, 7143-



7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)) may likewise be used.

It is also possible to transfer mutations in the respective genes or mutations relating to the expression of the  
5 relevant genes, by conjugation or transduction into various strains.

Furthermore for the production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae it may be advantageous in addition to  
10 the attenuation of the dgsA gene also to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide-adenine-dinucleotide phosphate.

15 The term "enhancement" describes in this connection the raising of the intracellular activity of one or more enzymes or proteins in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter or  
20 a gene that codes for a corresponding enzyme or protein having a high activity, and optionally by combining these measures.

By means of the aforementioned enhancement measures, in particular overexpression, the activity or concentration of  
25 the corresponding protein is in general raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most up to 1000% or 2000% referred to that of the wild type protein and/or the activity or concentration of the protein in the initial microorganism.

30 Thus, one or more of the genes selected from the following group may for example be simultaneously enhanced, in particular overexpressed:

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
  - the pyc gene coding for pyruvate carboxylase  
5 (DE-A-19 831 609),
  - the pps gene coding for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
  - the ppc gene coding for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
  - 10 • the genes pntA and pntB coding for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
  - the gene rhtB imparting homoserine resistance (EP-A-0 994 190),
  - the mqo gene coding for malate:quinone oxidoreductase  
15 (DE 100 348 33.5),
  - the gene rhtC imparting threonine resistance (EP-A-1 013 765), and
  - the thrE gene of Corynebacterium glutamicum coding for threonine export (DE 100 264 94.8).
- 20 The use of endogenous genes is in general preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is understood to mean the genes or nucleotide sequences present in the population of a species.

Furthermore for the production of L-amino acids, in  
25 particular L-threonine, it may be advantageous in addition to the attenuation of the dgsA gene also to attenuate, in particular to switch off or reduce the expression of one or more of the genes selected from the following group:

- the tdh gene coding for threonine dehydrogenase  
(Ravnikar and Somerville, Journal of Bacteriology 169,  
4716-4721 (1987)),
- the mdh gene coding for malate dehydrogenase (E.C.  
5 1.1.1.37) (Vogel et al., Archives in Microbiology 149,  
36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa  
(Accession Number AAC77180 of the National Center for  
Biotechnology Information (NCBI, Bethesda, MD, USA),
- 10 • the gene product of the open reading frame (orf) ytfP  
(Accession Number AAC77179 des National Center for  
Biotechnology Information (NCBI, Bethesda, MD, USA),
- the pckA gene coding for the enzyme phosphoenol pyruvate  
carboxykinase (Medina et al. (Journal of Bacteriology  
15 172, 7151-7156 (1990)),
- the poxB gene coding for pyruvate oxidase (Grabau and  
Cronan (Nucleic Acids Research 14 (13), 5449-5460  
(1986)),
- the fruR gene coding for the fructose repressor:  
20 (Jahreis et al., Molecular and General Genetics 226,  
332-336 (1991) and Accession No.: AE000118), and
- the aceA gene for isocitrate lyase (EC-No.: 4.1.3.1)  
kodierende (Matsuoko and McFadden; Journal of  
Bacteriology 170, 4528-4536 (1988) and Accession No.:  
25 AE000474)

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the dgsA gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of  
30 Amino Acid Producing Microorganisms", in: Overproduction of

Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention may be cultivated in a batch process (batch cultivation), in a  
5 fed batch process (feed process) or in a repeated fed batch process (repetitive feed process). A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart,  
10 1991)) or in the textbook by Storhas (Bioreaktoren and periphere Einrichtungen (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must appropriately satisfy the requirements of the respective strains. Descriptions  
15 of culture media of various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As carbon sources, sugars and carbohydrates such as for  
20 example glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats such as for example soya bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols  
25 such as for example glycerol and ethanol, and organic acids such as for example acetic acid, may be used. These substances may be used individually or as a mixture.

As nitrogen source, organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt  
30 extract, maize starch water, soya bean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate may be used. The nitrogen sources may be used individually or as a mixture.

As phosphorus source, phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used. The culture medium must furthermore contain salts of metals, such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Apart from these, suitable precursors may be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single batch or may be metered in in an appropriate manner during the cultivation.

In order to regulate the pH of the culture basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid are used as appropriate. In order to control foam formation antifoaming agents such as for example fatty acid polyglycol esters may be used. In order to maintain the stability of plasmids, suitable selectively acting substances, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for example air are fed into the culture. The temperature of the culture is normally 25°C to 45°C, and preferably 30°C to 40°C. Cultivation is continued until a maximum amount of L-amino acids (or L-threonine) has been formed. This target is normally achieved within 10 hours to 160 hours.

The L-amino acids may be analyzed by anion exchange chromatography followed by ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention can be used for the enzymatic production of L-amino acids, such as for example L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

- 5 A pure culture of the Escherichia coli K-12 strain DH5 $\alpha$ /pMAK705 was filed as DSM 13720 on 08 September 2000 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest Convention.
- 10 The present invention is described in more detail hereinafter with the aid of examples of implementation.

- The isolation of plasmid DNA from Escherichia coli as well as all techniques for the restriction, Klenow treatment and alkaline phosphatase treatment are carried out according to
- 15 Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). The transformation of Escherichia coli is, unless otherwise described, carried out according to Chung et al. (Proceedings of the National Academy of Sciences of the
- 20 United States of America, USA (1989) 86: 2172-2175).

The incubation temperature in the production of strains and transformants is 37°C. In the gene exchange process according to Hamilton et al., temperatures of 30°C and 44°C are used.

25 Example 1

Construction of the deletion mutation of the dgsA gene

- Parts of the gene regions lying upstream and downstream of the dgsA gene and parts of the 5'-region and 3'-region of the dgsA gene are amplified from Escherichia coli K12 using
- 30 the polymerase chain reaction (PCR) as well as synthetic oligonucleotides. Starting from the nucleotide sequence of the dgsA gene and sequences in E. coli K12 MG1655 (SEQ ID

No. 1, Accession Number AE000255) lying upstream and downstream, the following PCR primers are synthesised (MWG Biotech, Ebersberg, Germany):

dgsA'5'-1: 5' - CGAATGTAACGCTGGCTGAA - 3' (SEQ ID No. 3)

5 dgsA'5'-2: 5' - TCCAGCAATGGCAAGTCATC - 3' (SEQ ID No. 4)

dgsA'3'-1: 5' - CAGCACATCAGCGTTGAGAG - 3' (SEQ ID No. 5)

dgsA'3'-2: 5' - GATCGCCTGAGCTGTTAGCA - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated according to the manufacturer's instructions using  
10 "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A ca. 850 bp large DNA fragment from the 5'-region of the dgsA gene region (designated dgsA1) and a ca. 700 bp large DNA fragment from the 3'-region of the dgsA gene region (designated dgsA2) can be amplified with the specific  
15 primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with taq-DNA-polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are ligated according to the manufacturer's instructions in each case  
20 with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed in the E. coli strain TOP10F'. The selection of plasmid-carrying cells is carried out on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation  
25 the vector pCR2.1TOPOdgsA2 is cleaved with the restriction enzymes Ecl136II and XbaI, and the dgsA2 fragment after separation in 0.8% agarose gel is isolated using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After the plasmid DNA isolation the vector pCR2.1TOPOdgsA1  
30 is cleaved with the enzymes EcoRV and XbaI and ligated with the isolated dgsA2 fragment. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar to which 50 µg/ml of

ampicillin has been added. After the plasmid DNA isolation, those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 7 is present in cloned form are detected by control cleavage with the enzymes HindIII and XbaI. One of the plasmids is designated pCR2.1TOPO $\Delta$ dgsA.

#### Example 2

##### Construction of the exchange vector pMAK705 $\Delta$ dgsA

The dgsA allele described in Example 1 is isolated from the vector pCR2.1TOPO $\Delta$ dgsA after restriction with the enzymes HindIII and XbaI and separation in 0.8% agarose gel, and is ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622), that had been digested with the enzymes HindIII and XbaI. The ligation batch is transformed in DH5 $\alpha$  and plasmid-carrying cells are selected on LB agar to which 20  $\mu$ g/ml of chloramphenicol have been added. The successful cloning is detected after plasmid DNA isolation and cleavage with the enzymes HindIII and XbaI. The resultant exchange vector pMAK705 $\Delta$ dgsA (= pMAK705 $\Delta$ dgsA) is shown in Fig. 1.

#### Example 3

##### Site-specific mutagenesis of the dgsA gene in the E. coli strain MG442

The E. coli strain MG442 producing L-threonine is described in patent specification US-A- 4,278,765 and is filed as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For the exchange of the chromosomal dgsA gene by the plasmid-coded deletion construct, MG442 is transformed with the plasmid pMAK705 $\Delta$ dgsA. The gene exchange is carried out by the selection process described by Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622) and



is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with the following oligonucleotide primers:

dgSA'5'-1: 5' - CGAATGTAACGCTGGCTGAA - 3' (SEQ ID No. 3)

5 dgSA'3'-2: 5' - GATCGCCTGAGCTGTTAGCA - 3' (SEQ ID No. 6)

After the exchange the form of the  $\Delta$ dgSA allele shown in SEQ ID No. 8 is present in MG442. The strain obtained is designated MG442 $\Delta$ dgSA.

#### Example 4

#### 10 Production of L-threonine using the strain MG442 $\Delta$ dgSA

MG442 $\Delta$ dgSA is cultivated on minimal medium having the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose and 20 g/l agar. The formation of L-threonine is checked in batch  
15 cultures of 10 ml that are contained in 100 ml Erlenmeyer flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose are inoculated and incubated for 16 hours at  
20 37°C and 180 rpm in an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250  $\mu$ l of this preculture are reinoculated in 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$  and 20 g/l glucose) and  
25 incubated for 48 hours at 37°C. After incubation the optical density (OD) of the culture suspension is measured with an LP2W photometer from the Dr. Lange company (Dusseldorf, Germany) at a measurement wavelength of 660 nm.

30 The concentration of formed L-threonine is then determined in the sterile-filtered culture supernatant using an amino acid analyzer from Eppendorf-BioTrenik (Hamburg, Germany)

by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the test is given in Table 1.

Table 1

Strain	OD (660 nm)	L- threonine
MG442	6.0	1.5
MG442 $\Delta$ dgsA	6.5	1.8

5

Brief Description of the Figure:

- Fig. 1: pMAK705 $\Delta$ dgsA ( = pMAK705deltadgsA)

Length data are given as approximate values. The abbreviations and acronyms used have the following  
10 meanings:

- cat: chloramphenicol resistance gene
- rep-ts: temperature-sensitive replication region of the plasmid pSC101
- dgsA1: part of the 5'-region of the dgsA gene and of  
15 the upstream-lying region
- dgsA2: part of the 3'-region of the dgsA gene and of the downstream-lying region

The abbreviations for the restriction enzymes have the following meanings:

- 20 • BamHI: restriction endonuclease from *Bacillus amyloliquefaciens*

- BglIII: restriction endonuclease from *Bacillus globigii*
- ClaI: restriction endonuclease from *Caryophanon latum*
- EcoRI: restriction endonuclease from *Escherichia coli*
- 5 • EcoRV: restriction endonuclease from *Escherichia coli*
- HindIII: restriction endonuclease from *Haemophilus influenzae*
- KpnI: restriction endonuclease from *Klebsiella pneumoniae*
- 10 • PstI: restriction endonuclease from *Providencia stuartii*
- PvuI: restriction endonuclease from *Proteus vulgaris*
- SacI: restriction endonuclease from *Streptomyces achromogenes*
- 15 • SalI: restriction endonuclease from *Streptomyces albus*
- SmaI: restriction endonuclease from *Serratia marcescens*
- SphI: restriction endonuclease from *Streptomyces phaeochromogenes*
- 20 • SspI: restriction endonuclease from *Sphaerotilus species*
- XbaI: restriction endonuclease from *Xanthomonas badrii*
- 25 • XhoI: restriction endonuclease from *Xanthomonas holcicola*

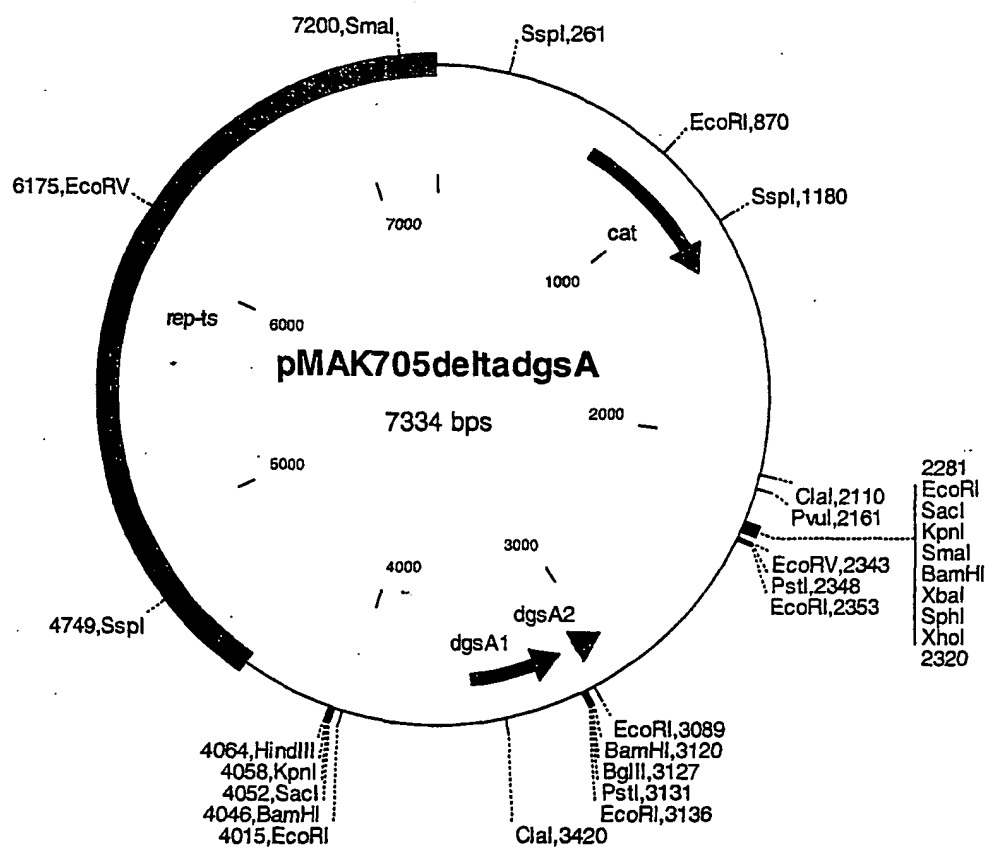
**What is Claimed is:**

1. Process for the production of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
  - 5 a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the dgsA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off,
  - 10 b) enrichment of the L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions  
15 thereof remain in the product.
2. Process according to claim 1, wherein microorganisms are used in which in addition further genes of the biosynthesis pathway of the desired L-amino acid are enhanced.
- 20 3. Process according to claim 1, wherein microorganisms are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.
4. Process according to claim 1, wherein the expression of  
25 the polynucleotide(s) that codes/code for the dgsA gene is attenuated, in particular is switched off.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide dgsA codes are  
30 reduced.

6. Process according to claim 1, wherein for the production of L-amino acids microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is enhanced, in particular overexpressed:
- 6.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene coding for pyruvate carboxylase,
- 6.3 the pps gene coding for phosphoenol pyruvate synthase,
- 6.4 the ppc gene coding for phosphoenol pyruvate carboxylase,
- 6.5 the genes pntA and pntB coding for transhydrogenase,
- 6.6 the gene rhtB imparting homoserine resistance,
- 6.7 the mqo gene coding for malate:quinone oxidoreductase,
- 6.8 the gene rhtC imparting threonine resistance, and
- 6.9 the thrE gene coding for threonine export.
7. Process according to claim 1, wherein for the production of L-amino acids microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is attenuated, in particular switched off, or the expression is reduced:
- 7.1 the tdh gene coding for threonine dehydrogenase,
- 7.2 the mdh gene coding for malate dehydrogenase.

- 7.3 the gene product of the open reading frame (orf) yjfA,
- 7.4 the gene product of the open reading frame (orf) ytfP,
- 5 7.5 the pckA gene coding for phosphoenol pyruvate carboxykinase,
- 7.6 the poxB gene coding for pyruvate oxidase,
- 7.7 the fruR gene coding for the fructose repressor,
- 7.8 the aceA gene coding for isocitrate lyase.

Fig. 1:



## SEQUENCE LISTING

5 <110> Degussa AG  
 <120> Process for the production of L-amino acids using strains of the family Enterobacteriaceae containing an attenuated dgsA-Gen  
 10 <130> 020003 BT  
 <160> 8  
 <170> PatentIn version 3.1  
 15 <210> 1  
 <211> 2306  
 <212> DNA  
 <213> Escherichia coli  
 20 <220>  
 <221> CDS  
 <222> (485)..(1705)  
 <223> dgsA gene  
 25 <400> 1  
 cgaatgtaac gctggctgaa ctggcgaaag aaccctttgt cttttttgat ccgcacgtcg 60  
 ggacaggggt gtatgacgat attctcgggc tgatgcgacg ttaccatttg acgcccgtca 120  
 30 tcaactcagga ggtgggagag gcaatgacca tcatcgggtct ggtttccgcc ggtctgggtg 180  
 tttcaatttt gcctgcgtca tttaaacgtg ttcagctcaa cgaaatgcgc tgggtgccga 240  
 ttgctgaaga ggatgcgggtt tctgaaatgt ggttggtctg gccgaaacat catgaacaaa 300  
 35 gtccggctgc gcgtaacttt cgtattcatc tgctgaatgc tctcaggtga gggaaatttc 360  
 agcgaaaaag cccgaaaaat gtgctgttaa tcacatgcct aagtaaaaat ttgacgacac 420  
 40 gtattgaagt gcttcaccat agcctacaga ttatttcgga gcgcgaaaat atagggagta 480  
 tgcg gtg gtt gct gaa aac cag cct ggg cac att gat caa ata aag cag 529  
 Met Val Ala Glu Asn Gln Pro Gly His Ile Asp Gln Ile Lys Gln  
 1 5 10 15  
 45 acc aac gcg ggc gcg gtt tat cgc ctg att gat cag ctt ggt cca gtc 577  
 Thr Asn Ala Gly Ala Val Tyr Arg Leu Ile Asp Gln Leu Gly Pro Val  
 20 25 30  
 50 tgc cgt atc gat ctt tcc cgt ctg gcg caa ctg gct cct gcc agt atc 625  
 Ser Arg Ile Asp Leu Ser Arg Leu Ala Gln Leu Ala Pro Ala Ser Ile  
 35 40 45  
 55 act aaa att gtc cgt gag atg ctc gaa gca cac ctg gtg caa gag ctg 673  
 Thr Lys Ile Val Arg Glu Met Leu Glu Ala His Leu Val Gln Glu Leu  
 50 55 60  
 60 gaa atc aaa gaa gcg ggg aac cgt ggc cgt ccg gcg gtg ggg ctg gtg 721  
 Glu Ile Lys Glu Ala Gly Asn Arg Gly Arg Pro Ala Val Gly Leu Val  
 65 70 75  
 gtt gaa act gaa gcc tgg cac tat ctt tct ctg cgc att agt cgc ggg 769  
 Val Glu Thr Glu Ala Trp His Tyr Leu Ser Leu Arg Ile Ser Arg Gly  
 80 85 90 95  
 65



	gag	att	ttc	ctt	gct	ctg	cgc	gat	ctg	agc	aaa	ctg	gtg	gtg	gaa	817	
	Glu	Ile	Phe	Leu	Ala	Leu	Arg	Asp	Leu	Ser	Ser	Lys	Leu	Val	Val	Glu	
				100						105				110			
5	gag	tcg	cag	gaa	ctg	gcg	tta	aaa	gat	gac	ttg	cca	ttg	ctg	gat	cgt	865
	Glu	Ser	Gln	Glu	Leu	Ala	Leu	Lys	Asp	Asp	Leu	Pro	Leu	Leu	Asp	Arg	
				115					120					125			
10	att	att	tcc	cat	atc	gat	cag	ttt	ttt	atc	cgc	cac	cag	aaa	aaa	ctt	913
	Ile	Ile	Ser	His	Ile	Asp	Gln	Phe	Phe	Ile	Arg	His	Gln	Lys	Lys	Leu	
			130					135					140				
15	gag	cgt	cta	act	tcg	att	gcc	ata	acc	ttg	ccg	gga	att	att	gat	acg	961
	Glu	Arg	Leu	Thr	Ser	Ile	Ala	Ile	Thr	Leu	Pro	Gly	Ile	Ile	Asp	Thr	
		145					150					155					
20	gaa	aat	ggc	att	gta	cat	cgc	atg	ccg	ttc	tac	gag	gat	gta	aaa	gag	1009
	Glu	Asn	Gly	Ile	Val	His	Arg	Met	Pro	Phe	Tyr	Glu	Asp	Val	Lys	Glu	
	160					165					170					175	
25	atg	ccg	ctc	ggc	gag	gcg	ctg	gag	cag	cat	acc	ggc	gtt	ccg	gtt	tat	1057
	Met	Pro	Leu	Gly	Glu	Ala	Leu	Glu	Gln	His	Thr	Gly	Val	Pro	Val	Tyr	
				180						185					190		
30	att	cag	cat	gat	atc	agc	gca	tgg	acg	atg	gca	gag	gcc	ttg	ttt	ggc	1105
	Ile	Gln	His	Asp	Ile	Ser	Ala	Trp	Thr	Met	Ala	Glu	Ala	Leu	Phe	Gly	
				195					200					205			
35	gcc	tca	cgc	ggg	gcg	cgc	gat	gtg	att	cag	gtg	gtt	atc	gat	cac	aac	1153
	Ala	Ser	Arg	Gly	Ala	Arg	Asp	Val	Ile	Gln	Val	Val	Ile	Asp	His	Asn	
			210					215					220				
40	gtg	ggg	gcg	ggc	gtc	att	acc	gat	ggc	cat	ctg	cta	cac	gca	ggc	agc	1201
	Val	Gly	Ala	Gly	Val	Ile	Thr	Asp	Gly	His	Leu	Leu	His	Ala	Gly	Ser	
		225					230					235					
45	agt	agt	ctc	gtg	gaa	ata	ggc	cac	aca	cag	gtc	gac	ccg	tat	ggg	aaa	1249
	Ser	Ser	Leu	Val	Glu	Ile	Gly	His	Thr	Gln	Val	Asp	Pro	Tyr	Gly	Lys	
	240					245					250				255		
50	cgc	tgt	tat	tgc	ggg	aat	cac	ggc	tgc	ctc	gaa	acc	atc	gcc	agc	gtg	1297
	Arg	Cys	Tyr	Cys	Gly	Asn	His	Gly	Cys	Leu	Glu	Thr	Ile	Ala	Ser	Val	
				260					265						270		
55	gac	agt	att	ctt	gag	ctg	gca	cag	ctg	cgt	ctt	aat	caa	tcc	atg	agc	1345
	Asp	Ser	Ile	Leu	Glu	Leu	Ala	Gln	Leu	Arg	Leu	Asn	Gln	Ser	Met	Ser	
				275					280					285			
60	tcg	atg	tta	cat	gga	caa	ccg	tta	acc	gtg	gac	tca	ttg	tgt	cag	gcg	1393
	Ser	Met	Leu	His	Gly	Gln	Pro	Leu	Thr	Val	Asp	Ser	Leu	C			

ttc ccg gtc atc tca gac agc atc cgt cag cag gcc ctt cct gcg tat 1585  
 Phe Pro Val Ile Ser Asp Ser Ile Arg Gln Gln Ala Leu Pro Ala Tyr  
 355 360 365

5 agt cag cac atc agc gtt gag agt act cag ttt tct aac cag ggc acg 1633  
 Ser Gln His Ile Ser Val Glu Ser Thr Gln Phe Ser Asn Gln Gly Thr  
 370 375 380

10 atg gca ggc gct gca ctg gta aaa gac gcg atg tat aac ggt tct ttg 1681  
 Met Ala Gly Ala Ala Leu Val Lys Asp Ala Met Tyr Asn Gly Ser Leu  
 385 390 395

15 ttg att cgt ctg ttg cag ggt taa ctttttttaa ctgttctacc aaaatttgcg 1735  
 Leu Ile Arg Leu Leu Gln Gly  
 400 405

ctatctcaat ttgggccagg aaagcataac ttagactttc aagggttaatt attttcctgg 1795

20 tttatatattg tgaagcataa cgggtggagtt agtgatgctg aagcgtttct ttattaccgg 1855  
 tacagacact tctgtaggga aaacggtggt ttcccgcgca ttgtacaag cgttagcctc 1915  
 ccagggaaaa acggttgccg gatataaacc cgtagcgaag gggagcaaag agacacccga 1975

25 agggctgcgt aataaagatg ccctggtggt gcagagtgtt tcaaccatcg aactgcctta 2035  
 tgaagcagtt aatcctatcg cgtaagcga agaagaaagt agcgtggcgc acagttgccc 2095  
 aatcaattac accctcattt caaacggcct ggcaaacctg accgaaaaag tcgatcatgt 2155

30 cgtggtagaa gggactggcg gctggcgag tctgatgaat gatttgcgtc cactctctga 2215  
 atgggtagtg caggaacaac tgccggtggt gatggtgtc ggtattcagg aaggttgcac 2275

35 taaccatgca ctgctaacag ctcaggcgat c 2306

<210> 2  
 <211> 406  
 40 <212> PRT  
 <213> Escherichia coli

<400> 2  
 45 Met Ala Glu Asn Gln Pro Gly His Ile Asp Gln Ile Lys Gln Thr 15  
 1 5 10 15

Asn Ala Gly Ala Val Tyr Arg Leu Ile Asp Gln Leu Gly Pro Val Ser  
 20 25 30

50 Arg Ile Asp Leu Ser Arg Leu Ala Gln Leu Ala Pro Ala Ser Ile Thr  
 35 40 45

Lys Ile Val Arg Glu Met Leu Glu Ala His Leu Val Gln Glu Leu Glu  
 50 55 60

55 Ile Lys Glu Ala Gly Asn Arg Gly Arg Pro Ala Val Gly Leu Val Val  
 65 70 75 80

60 Glu Thr Glu Ala Trp His Tyr Leu Ser Leu Arg Ile Ser Arg Gly Glu  
 85 90 95

Ile Phe Leu Ala Leu Arg Asp Leu Ser Ser Lys Leu Val Val Glu Glu  
 100 105 110

65 Ser Gln Glu Leu Ala Leu Lys Asp Asp Leu Pro Leu Leu Asp Arg Ile

	115	120	125
	Ile Ser His Ile Asp Gln Phe Phe Ile Arg His Gln Lys Lys Leu Glu		
	130	135	140
5	Arg Leu Thr Ser Ile Ala Ile Thr Leu Pro Gly Ile Ile Asp Thr Glu		
	145	150	155
	Asn Gly Ile Val His Arg Met Pro Phe Tyr Glu Asp Val Lys Glu Met		
10		165	170
	Pro Leu Gly Glu Ala Leu Glu Gln His Thr Gly Val Pro Val Tyr Ile		
	180	185	190
15	Gln His Asp Ile Ser Ala Trp Thr Met Ala Glu Ala Leu Phe Gly Ala		
	195	200	205
	Ser Arg Gly Ala Arg Asp Val Ile Gln Val Val Ile Asp His Asn Val		
20		215	220
	Gly Ala Gly Val Ile Thr Asp Gly His Leu Leu His Ala Gly Ser Ser		
	225	230	235
25	Ser Leu Val Glu Ile Gly His Thr Gln Val Asp Pro Tyr Gly Lys Arg		
	245	250	255
	Cys Tyr Cys Gly Asn His Gly Cys Leu Glu Thr Ile Ala Ser Val Asp		
	260	265	270
30	Ser Ile Leu Glu Leu Ala Gln Leu Arg Leu Asn Gln Ser Met Ser Ser		
	275	280	285
	Met Leu His Gly Gln Pro Leu Thr Val Asp Ser Leu Cys Gln Ala Ala		
35		295	300
	Leu Arg Gly Asp Leu Leu Ala Lys Asp Ile Ile Thr Gly Val Gly Ala		
	305	310	315
40	His Val Gly Arg Ile Leu Ala Ile Met Val Asn Leu Phe Asn Pro Gln		
	325	330	335
	Lys Ile Leu Ile Gly Ser Pro Leu Ser Lys Ala Ala Asp Ile Leu Phe		
	340	345	350
45	Pro Val Ile Ser Asp Ser Ile Arg Gln Gln Ala Leu Pro Ala Tyr Ser		
	355	360	365
	Gln His Ile Ser Val Glu Ser Thr Gln Phe Ser Asn Gln Gly Thr Met		
50		375	380
	Ala Gly Ala Ala Leu Val Lys Asp Ala Met Tyr Asn Gly Ser Leu Leu		
	385	390	395
55	Ile Arg Leu Leu Gln Gly		
	405		
	<210> 3		
	<211> 20		
	<212> DNA		
60	<213> Artificial sequence		
	<220>		
	<221> Primer		
	<222> (1)..(20)		

```

    <400> 3
5    cgaatgtaac gctggctgaa                                20
    <210> 4
    <211> 20
    <212> DNA
    <213> Artificial sequence
10
    <220>
    <221> Primer
    <222> (1)..(20)
    <223> dgsA `5`-2
15
    <400> 4
    tccagcaatg gcaagtcac                                20
    <210> 5
    <211> 20
    <212> DNA
    <213> Artificial sequence
    <220>
25    <221> Primer
    <222> (1)..(20)
    <223> dgsA `3`-1
    <400> 5
30    cagcacatca gcgttgagag                                20
    <210> 6
    <211> 20
    <212> DNA
35    <213> Artificial sequence
    <220>
    <221> Primer
    <222> (1)..(20)
40    <223> dgsA `3`-2
    <400> 6
    gatcgccctga gctgttagca                                20
45    <210> 7
    <211> 1756
    <212> DNA
    <213> Escherichia coli
50    <220>
    <221> misc_feature
    <222> (1)..(1756)
    <223>
55    <220>
    <221> misc_feature
    <222> (1)..(60)
    <223> Technical DNA/ remainder polylinker sequence
60    <220>
    <221> misc_feature
    <222> (61)..(921)
    <223> Part of the upstream-lying region and part of the 5'-region
65    of the dgsA gene

```

<220>  
 <221> misc\_feature  
 <222> (922)..(986)  
 <223> Technical DNA/ remainder polylinker sequence  
 5  
 <220>  
 <221> misc\_feature  
 <222> (987)..(1704)  
 <223> Part of the 3'-region of the dgsA gene and part of the downstream-  
 10 lying region  
 <220>  
 <221> misc\_feature  
 <222> (1705)..(1756)  
 15 <223> Technical DNA/ remainder polylinker sequence  
 <400> 7  
 agcttggtac cgagctcgga tccactagta acggccgccga gtgtgctgga attcgcctt 60  
 20 cgaatgtaac gctggctgaa ctggcgaaag aaccctttgt cttttttgat ccgcacgtcg 120  
 ggacagggct gtatgacgat attctcgggc tgatgcgacg ttaccatttg acgcccgtca 180  
 25 tcaactcagga ggtgggag gcaatgacca tcacgggtct ggtttccgcc ggtctgggtg 240  
 tttcaatttt gcctgcgtca tttaaactgt ttcagctcaa cgaaatgcgc tgggtgccga 300  
 ttgctgaaga ggatgcggtt tctgaaatgt ggttggtctg gccgaaacat catgaacaaa 360  
 30 gtccggctgc gcgtaacttt cgtattcatc tgctgaatgc tctcaggtga gggaaatttc 420  
 agcgaaaaag cccgaaaaat gtgctgttaa tcacatgcct aagtaaaaat ttgacgacac 480  
 35 gtattgaagt gcttcacatc agcctacaga ttatttcgga gcgcgaaaat atagggagta 540  
 tgccggtggt gctgaaaacc agcctgggca cattgatcaa ataaagcaga ccaacgcggg 600  
 cgccggttat cgctgattg atcagcttgg tccagtctcg cgtatcgatc tttcccgctc 660  
 40 ggccgcaactg gctcctgccg gtatcactaa aattgtccgt gagatgctcg aagcacacct 720  
 ggtgcaagag ctggaaatca aagaagcggg gaaccgtggc cgtccggcgg tggggtggt 780  
 45 ggttgaaact gaagcctggc actatctttc tctgcgcatt agtcgcgggg agattttcct 840  
 tgctctgcgc gatctgagca gcaaactggt ggtggaagag tcgcaggaac tggcgttaaa 900  
 agatgacttg ccattgctgg aaagggcgaa ttctgcagat ctccgatcca ctagtaacgg 960  
 50 ccgccagtgt gctggaattc gcccttcagc acatcagcgt tgagagtact cagttttcta 1020  
 accagggcac gatggcaggc gctgcactgg taaaagacgc gatgtataac ggttctttgt 1080  
 55 tgattcgtct gttgcagggt taacattttt taactgttct accaaaattt gcgctatctc 1140  
 aatttggggc aggaaagcat aacttagact ttcaagggtta attattttcc tggtttatat 1200  
 ttgtgaagca taacggtgga gttagtgtg ctgaagcgtt tctttattac cggtagacac 1260  
 60 acttctgtag ggaaaacggt ggtttccgcg gcattgctac aagcgtttag ctcccaggga 1320  
 aaaacgggtt cgggatataa acccgtagcg aaggggagca aagagacacc cgaagggctg 1380  
 65 cgtaataaag atgccttggg gttgcagagt gtttcaacca tcgaactgcc ttatgaagca 1440

```

      gttaatccta tcgcgttaag cgaagaagaa agtagcgtgg cgcacagttg cccaatcaat 1500
      tacaccctca tttcaaacgg cctggcaaac ctgaccgaaa aagtcgatca tgctcgtgga 1560
5    gaagggactg gcggctggcg cagtctgatg aatgatttgc gtccactctc tgaatgggta 1620
      gtgcaggaac aactgccggt gttgatggtt gtcggtattc aggaagggtg cattaaccat 1680
      gcactgctaa cagctcaggc gatcaagggc gaattctgca gatatccatc acactggcgg 1740
10   ccgctcgagc atgcat 1756

      <210> 8
      <211> 559
15   <212> DNA
      <213> Escherichia coli

      <220>
      <221> misc_feature
20   <222> (1)..(559)
      <223>

      <220>
      <221> misc_feature
25   <222> (1)..(3)
      <223> Start codon of the delta dgsA allele

      <220>
      <221> misc_feature
30   <222> (1)..(377)
      <223> 5'-region of the delta dgsA allele

      <220>
      <221> misc_feature
35   <222> (378)..(442)
      <223> Technical DNA/ remainder polylinker sequence

      <220>
      <221> misc_feature
40   <222> (443)..(556)
      <223> 3'-region of the delta dgsA allele

      <220>
      <221> misc_feature
45   <222> (557)..(559)
      <223> Stop codon of the delta dgsA allele

      <400> 8
50   gtggttgctg aaaaccagcc tgggcacatt gatcaaataa agcagaccaa cgcgggcgcg 60
      gtttatcgcc tgattgatca gcttggtcca gtctcgcgta tcgatctttc ccgtctggcg 120
      caactggctc ctgccagtat cactaaaatt gtccgtgaga tgctcgaagc acacctgggtg 180
55   caagagctgg aatcaaaga agcggggaac cgtggccgct cggcggtggg gctggtgggtt 240
      gaaactgaag cctggcacta tctttctctg cgcattagtc gcggggagat tttccttgct 300
      ctgcgcgatc tgagcagcaa actggtggtg gaagagtcgc aggaactggc gttaaaagat 360
60   gacttgccat tgctggaaaag ggcgaattct gcagatctcg gatccactag taacggccgc 420
      cagtgtgctg gaattcgccc ttcagcacat cagcggttag agtactcagt tttctaacca 480
65   gggcacgatg gcaggcgctg cactggtaaa agacgcgatg tataacgggt ctttgttgat 540

```

tcgtctgttg cagggtaa

559

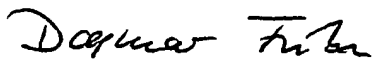
BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

PCT/EP02/02419

INTERNATIONAL FORM

Degussa-Hüls AG  
Kantstr. 2  
33790 Halle

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DH5 $\alpha$ /pMAK705	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 13720
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by:  <input checked="" type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-09-08 (Date of the original deposit) <sup>1</sup> .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):    Date: 2000-09-12

<sup>1</sup> Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.



BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE


PCT/EP02/02419

INTERNATIONAL FORM

Degussa-Hüls AG  
Kantstr. 2  
33790 Halle

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa-Hüls AG Kantstr. 2 Address: 33790 Halle	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720  Date of the deposit or the transfer <sup>1</sup> : 2000-09-08
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2000-09-08 <sup>2</sup> . On that date, the said microorganism was  <input checked="" type="checkbox"/> viable  <input type="checkbox"/> no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED <sup>3</sup>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):    Date: 2000-09-12

<sup>1</sup> Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

<sup>2</sup> In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

<sup>3</sup> Mark with a cross the applicable box.

<sup>4</sup> DSM is the International Depositary Authority and the number of the test was received.

**(57) Abstract:** The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) selection of a suitable bacterial strain, b) cultivation of the bacterial strain in a medium containing the L-amino acid, c) isolation of the L-amino acid from the medium or from the cells of the bacteria, and d) isolation of the L-amino acid.

# INTERNATIONAL SEARCH REPORT

Inter Application No  
PC/ cr 02/02419

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C12P13/08 C12P13/04 //(C12P13/08,C12R1:19)		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12P C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 278 765 A (DEBABOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14) cited in the application the whole document ---	1-7
A	MORRIS P W ET AL.: "Cloning and location of the dgsA gene of Escherichia coli." JOURNAL OF BACTERIOLOGY, vol. 163, no. 2, August 1985 (1985-08), pages 785-786, XP008016939 ISSN: 0021-9193 cited in the application the whole document --- -/--	1-7
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : 'A' document defining the general state of the art which is not considered to be of particular relevance 'E' earlier document but published on or after the international filing date 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 'O' document referring to an oral disclosure, use, exhibition or other means 'P' document published prior to the international filing date but later than the priority date claimed 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. '&' document member of the same patent family		
Date of the actual completion of the international search 20 May 2003		Date of mailing of the international search report 02/06/2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016		Authorized officer van de Kamp, M

## INTERNATIONAL SEARCH REPORT

Intel Application No  
PC 1/EP 02/02419

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HOSONO K ET AL.: "Decreasing accumulation of acetate in a rich medium by Escherichia coli on introduction of genes on a multicopy plasmid."  BIOSCIENCE, BIOTECHNOLOGY, AND BIOCHEMISTRY,  vol. 59, no. 2, February 1995 (1995-02),  pages 256-261, XP001148978  ISSN: 0916-8451  cited in the application  abstract  page 260, left-hand column, line 38 -page  261, left-hand column, line 36</p>	1-7
A	<p>WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG)  21 October 1999 (1999-10-21)  page 5, line 20-24  examples 4,7,9,10  claims 41,49  figures 1,4</p>	1-7
A	<p>EP 0 643 135 A (AJINOMOTO KK)  15 March 1995 (1995-03-15)  the whole document</p>	1-7
A	<p>EP 0 237 819 A (KYOWA HAKKO KOGYO KK)  23 September 1987 (1987-09-23)  the whole document</p>	1-7
A	<p>DATABASE WPI  Section Ch, Week 199148  Derwent Publications Ltd., London, GB;  Class B05, AN 1991-351136  XP002241222  &amp; JP 03 236786 A (KYOWA HAKKO KOGYO KK),  22 October 1991 (1991-10-22)  abstract</p>	1-7
A	<p>MICHAL G: "Biochemical pathways: an atlas of biochemistry and molecular biology"  1999, JOHN WILEY &amp; SONS INC. AND SPEKTRUM  AKADEMISCHER VERLAG, NEW YORK -  HEIDELBERG XP002240848  ISBN: 0-471-33130-9  figures 4.2-1, 4.5-1 and 4.5-2  paragraph '4.5.3!'</p>	1-7

-/--

# INTERNATIONAL SEARCH REPORT

Intel Application No  
PCI/EP 02/02419

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JETTEN M S M ET AL.: "Recent advances in the physiology and genetics of amino acid-producing bacteria." CRC CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 15, no. 1, 1995, pages 73-103, XP000613291 ISSN: 0738-8551 page 82, left-hand column, line 31-43 page 90, left-hand column, line 1 -page 92, left-hand column, line 17 ---	1-7
A	KRAEMER R: "Genetic and physiological approaches for the production of amino acids" JOURNAL OF BIOTECHNOLOGY, vol. 45, no. 1, 1996, pages 1-21, XP002178648 ISSN: 0168-1656 the whole document ---	1-7
A	SAWERS G: "The anaerobic degradation of L-serine and L-threonine in enterobacteria: networks of pathways and regulatory signals" ARCHIVES OF MICROBIOLOGY, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document ---	1-7
E	WO 02 081698 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 21 -page 10, line 22 claim 7 ---	1-7
E	WO 02 081722 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 21 -page 10, line 22 claim 7 -----	1-7

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Integ

Application No

PCT/EP 02/02419

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4278765	A	14-07-1981	SU 875663 A1 HU 190999 B	15-09-1982 28-12-1986
WO 9953035	A	21-10-1999	AU 3555999 A BR 9909615 A CA 2325598 A1 EP 1073722 A1 JP 2002511250 T WO 9953035 A1 US 2003087381 A1 US 6455284 B1	01-11-1999 12-12-2000 21-10-1999 07-02-2001 16-04-2002 21-10-1999 08-05-2003 24-09-2002
EP 0643135	A	15-03-1995	AT 203769 T CZ 9401658 A3 DE 69330518 D1 DE 69330518 T2 DK 643135 T3 EP 0643135 A1 JP 3331472 B2 SK 81994 A3 US 5661012 A EP 1020526 A2 ES 2158867 T3 WO 9411517 A1 RU 2113484 C1	15-08-2001 15-12-1994 06-09-2001 08-05-2002 15-10-2001 15-03-1995 07-10-2002 10-05-1995 26-08-1997 19-07-2000 16-09-2001 26-05-1994 20-06-1998
EP 0237819	A	23-09-1987	DE 3788583 D1 DE 3788583 T2 EP 0237819 A2 JP 2574786 B2 JP 63273487 A KR 9108634 B1 US 5017483 A	10-02-1994 19-05-1994 23-09-1987 22-01-1997 10-11-1988 19-10-1991 21-05-1991
JP 3236786	A	22-10-1991	JP 2877414 B2	31-03-1999
WO 02081698	A	17-10-2002	DE 10116518 A1 WO 02081721 A2 WO 02081698 A2 WO 02081722 A2 US 2003054503 A1 US 2003059903 A1	17-10-2002 17-10-2002 17-10-2002 17-10-2002 20-03-2003 27-03-2003
WO 02081722	A	17-10-2002	DE 10116518 A1 WO 02081721 A2 WO 02081698 A2 WO 02081722 A2 US 2003054503 A1 US 2003059903 A1	17-10-2002 17-10-2002 17-10-2002 17-10-2002 20-03-2003 27-03-2003

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☒ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**